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Effect of River Ecological Restoration on Biofilm Microbial Community Composition

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Abstract: Across the world, there have been increasing attempts to restore good ecological condition to degraded rivers through habitat restoration. Microbial communities developing as biofilms play an important role in river ecosystem functioning by driving organic matter decomposition and ecosystem respiration. However, little is known about the structure and function of microbial communities in riverine systems and how these change when habitat restoration is implemented. Here, we compared the biofilm bacterial community composition using 16S rRNA genes targeted high-throughput Illumina Miseq sequencing in three river types, degraded urban rivers, urban rivers undergoing habitat restoration and forested rivers (our reference conditions). We aimed to determine: (i) the biofilm bacterial community composition affected by habitat restoration (ii) the difference in bacterial diversity in restored rivers, and (iii) correlations between environmental variables and bacterial community composition. The results showed that both water quality and biofilm bacterial community structure were changed by habitat restoration. In rivers where habitat had been restored, there was an increase in dissolved oxygen, a reduction in organic pollutants, a reduction in bacterial diversity and a related developing pattern of microbial communities, which is moving towards that of the reference conditions (forested rivers). River habitat management stimulated the processing of organic pollutants through the variation in microbial community composition, however, a big difference in bacterial structure still existed between the restored rivers and the reference forest rivers. Thus, habitat restoration is an efficient way of modifying the biofilm microbial community composition for sustainable freshwater management. It will, however, take a much longer time for degraded rivers to attain a similar ecosystem quality as the "pristine" forest sites than the seven years of restoration studied here.

Keywords: bacterial community; biofilm; Illumina Miseq sequencing; habitat restoration; river ecosystem

1. Introduction

One of the current aims in riverine ecology is to use ecological restoration techniques to improve the quality of river ecosystem health, especially in urban areas where rivers have often been degraded severely [1]. Degraded rivers are normally formed by water pollution, land reclamation, dredging, channelization, altered hydrology and the clearing of riparian zones [2,3]. Ecological restoration approach aims to recover river habitat quality by increasing river habitat complexity and heterogeneity;

this is achieved by reconfiguring the river channel, increasing flood plain areas, adding in-stream islands, and aquatic vegetation [1]; all designed to enhance the hydraulic and substrate heterogeneity and macrophyte colonization. In combination, these treatments should increase food availability within the ecosystem [4,5], and eventually, a complexity of aquatic habitats (e.g., riffle, run, pool, and debris dam classifications) will develop in these restored rivers [6].

Healthy river habitats not only allow the living micro-organisms, aquatic flora (e.g., algae, aquatic plants) and fauna (e.g., macro-invertebrates, fishes) to persist, but they can also provide important ecosystems services, for example by reducing pollutants, such as organic matter, nutrients and heavy metals [7]. Riverine habitats are known to influence the diversity and composition of aquatic biotas through river morphology, hydrology, sedimentation, and by changing environmental variables at the reach scale, the latter important for larger stream organisms such as fish and macro-invertebrates [8]. For example, the surface features of the stream may influence detritus accumulation [9], and hence form 'refuges' for predators [10,11]. Moreover, the habitat complexity generated by surface irregularities exerts a significant impact on the abundance and diversity of benthic invertebrates in stream systems [6,12–14]. In a meta-analysis, in-stream habitat heterogeneity restoration (including wood, boulder additions and channel reconfigurations) enhanced macro-invertebrate richness [6]. Nuttle et al., (2017) also found that cutting gates, restoring substrates, and enhancing in-stream and riparian habitats, significantly enhanced (i) the taxon richness of macro-invertebrates, and (ii) the richness and abundance of fish in 18 mitigation sites [15]. In spite of this, very little is known about the effects of river habitat restoration on the composition of biofilm microbial communities.

Biofilms are a complex assemblage of microbial communities composed of bacteria, archaea, fungi, algae, and exopolysaccharides produced by the microorganisms. They are important components of stream ecosystems and are considered a good bioindicator of environmental health [16], not only because of their high abundance in most natural environments but also because of their sensitivity to environmental changes with short life cycle. Biofilms are a basic component of freshwater food webs; they adhere to the surfaces of rock particles and aquatic plants and are influenced by many environmental factors including temperature, light, shear forces, nutrients and contaminants [17–19]. They fix energy and carbon by photosynthesis and chemosynthesis and some can also fix nitrogen [20]. They also recycle organic nitrogen, impact on dissolved organic matter, and play key roles in nutrient cycling, organic compound degradation, water quality remediation and suspended sediment removal [21]. Effectively, altering any environmental factor can affect stream biofilm communities, and this may, in turn, alter their function of the whole stream ecosystem [22]. Bacteria are an indispensable part of the epilithic biofilm, usually occupying 1%–5% of the epilithic biofilm, and playing key roles in nutrient cycling, metabolic processes and many other biogeochemical processes and ecosystem functions [23–25]. The rates of bacterial-mediated nitrification, denitrification, and heterotrophic nitrogen (N) uptake in small streams have been shown to affect downstream water quality [25–27]. However, the impact of habitat restoration on biofilm bacterial community composition is still unclear.

To address this lack of information about biofilms during riverine restoration, we compared microbial populations in three different river types along a disturbance gradient. The most disturbed sites in this study were in urban areas, and the least disturbed sites were in forested catchments. In between, were rivers in urban areas where the habitat had been restored within the last seven years as part of an ecological restoration strategy. We measured a range of environmental factors and assessed the microbial community using a standardized field procedure followed by 16S rRNA Illumina MiSeq. Through comparing the relationship among habitat status, environmental parameters and bacterial community composition, we aimed to determine: (i) the biofilm bacterial community composition affected by habitat restoration (ii) the difference in bacterial diversity in restored rivers and urban degraded rivers, and (iii) any correlations between bacterial community composition and selected environmental variables. We hypothesized that habitat restoration would alter the biofilm bacterial community composition in these restored rivers compared to the degraded ones and that they

would become similar to the reference forest rivers. The bacterial diversity would be shifted toward a near-natural state where habitat had been restored. The substrate composition and physico-chemical variables like dissolved oxygen, nutrients and organic pollutants might be leading factors affecting the bacterial community composition in river groups.

2. Materials and Methods

2.1. Study Sites

This study compared three stream types in the winter of 2017: (i) degraded rivers in urban areas, (ii) restored rivers, where an aquatic habitat restoration scheme had been implemented within the last seven years for each river; (iii) rivers in forested catchments as reference conditions. Nine streams with similar-sized watersheds within the Anji City Region, Zhejiang Province PRC were selected for this study (Figure 1, Supplementary material Table S1). There were three replicates of each stream type, all located in different places in Anji City. The average day/night temperatures of the region were 12 °C/5 °C in winter, and average precipitation of 50 mm.

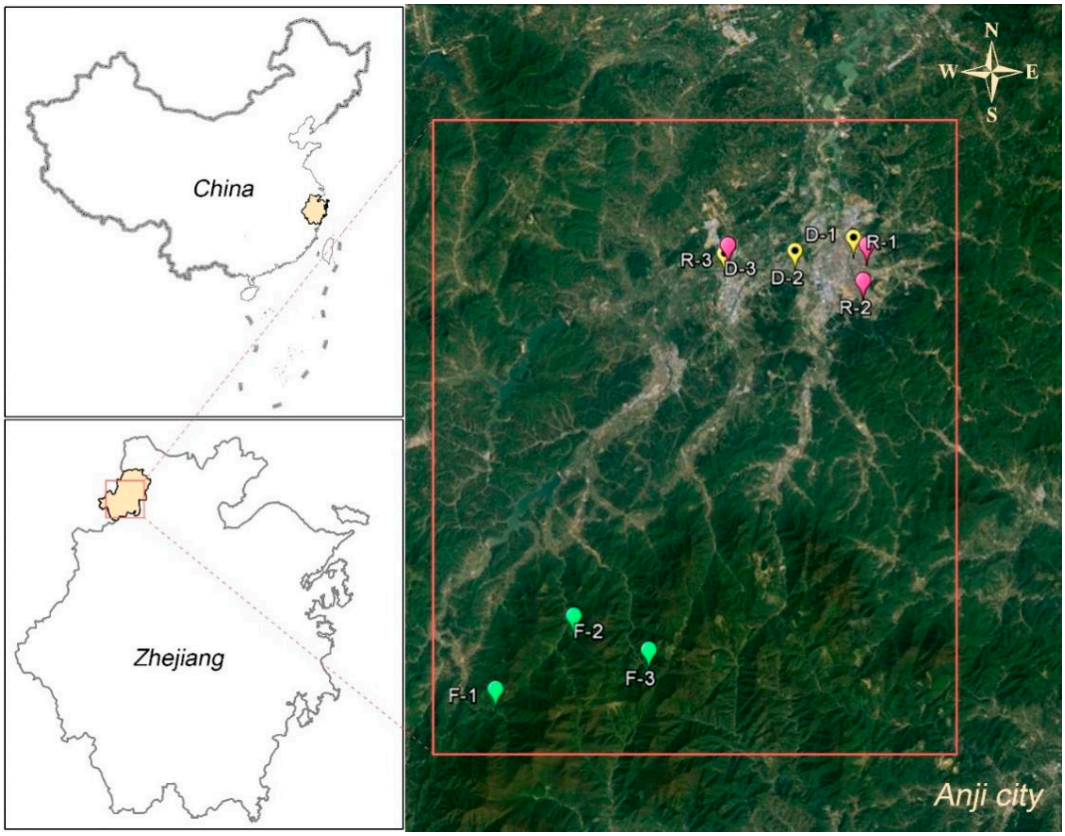

Figure 1. Location of the sampling sites within the Anji City Region, People's Republic of China (PRC), containing three degraded urban rivers (D), three restored rivers (R) and three Forested rivers (F). The three forest streams (F) were upstream from Anji City; the three restored rivers (R) and the three degraded urban rivers (D) were downstream of the forest ones.

The three urban degraded sites (denoted D) were similar to the pre-restoration status of our restored rivers, Tongxin River is located in the city center, and the other two are located in the suburban districts. The three restored rivers (denoted R) have been restored for up to seven years using a mixture of ecological restoration techniques to reconstruct a natural river form. The techniques used included channel re-meandering, creation of riffles, pools and run areas, construction of floating islands, aquatic plant re-introduction, and riparian zone afforestation. A subsidiary aim was to provide ecosystems

that could be used for ecological research, education and entertainment. Three forest streams (denoted F) were in the Tianmu Mountains (maximum elevation 590 m), 40-km upstream from Anji City were set as our "reference" conditions because pristine rivers were not available in the city area. There has been relatively little human interference on these forest streams, and they represent pre-urban landscape form where the urban rivers have derived [28].

2.2. Habitat Survey and Physico-Chemical Parameters of Stream Water

Habitat surveys were performed in December 2017 and January 2018. Reach canopy cover was estimated visually and the presence of various mesohabitat counted (island, pool, riffle). To estimate the variation of sediment grain size within each reach studied, 100 sediment particles were selected randomly on the river bed and proportions of boulders (>256 mm in diameter), cobbles (64–256 mm), pebbles (4–64 mm) and sand grains (2–4 mm) were counted [29]. The substrate diversity was calculated using the percentage cover of all substrate classes using the Shannon diversity index H' [30] for each study site.

Thereafter, within each river, the river width was measured using a 100 m tape. Water velocity and river depth were measured at five evenly-spaced points across the channel using Teledyne flow meters (ISCO, Lincoln, NE, USA) and a steel ruler. Water quality in each river was monitored at three different points with 3 m interval at the maximum by in situ measurements of temperature, pH, both using a HACH pH/temperature meter (LA-pH 10, HACH, Loveland, CO, USA), dissolved oxygen (DO), using a YSI Professional Plus probe (YSI Pro Plus, YSI, Yellow Springs, OH, USA), and turbidity, using a turbidity meter (DR2100Q, HACH, Loveland, CO, USA). One liter of water sample was collected from each stream and filtered in the field through 0.45 µm Jingteng syringe tip filters and preserved at 4 °C before sending to the laboratory. These water samples were analyzed within 48 h for (i) total nitrogen (TN) and total organic carbon (TOC), measured using a total organic carbon analyzer with a total nitrogen module (Multi N/C3100, Analytik Jena, Jena, Germany), (ii) ammonium nitrogen (NH_4-N), nitrate-nitrogen (NO_3-N), and total phosphorus (TP), measured using a QuickChem® Flow Injection Analysis system (Lachat Instrument, HACH, Loveland, CO, USA), and (iii) chemical oxygen demand (COD), measured using a DR1010 COD analyzer (HACH, Loveland, CO, USA).

2.3. Biofilm Sampling Procedure

Biofilm was sampled by placing four 10 cm × 10 cm autoclaved unglazed tiles, at 0.3 m water depth in each river for 39 days; thereafter the biofilms were collected by scraping the accumulated materials from the tiles into 50 mL tubes covered with aluminum foil, and transported in a cool box to the laboratory. The material in each 50 mL tube was then separated into two, one part was filtered through 0.45 µm membrane filter (Jingteng) to measure chlorophyll *a* (Chl-*a*) using a fluorimeter (10AU, Turner Designs, Sunnyvale, CA, USA) after acetone extraction [31], and the other part was filtered on 0.22 µm pore size polycarbonate membrane filters (Millipore, MA, USA) using a vacuum pump; these filtrates were stored in sterile Petri dishes at −20 °C until DNA extraction.

2.4. DNA Extraction and Analysis of Bacterial Community Composition

The genomic DNA of all the biofilm samples was extracted using DNA extraction Kit (MO BIO PowerBiofilm® DNA Isolation Kit, MO BIO Laboratories, Carlsbad, CA, USA) based on a standard protocol. The DNA concentration was quantified using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA), and the ratio of absorbance at 260 nm and 280 nm was checked to ensure the quality of DNA obtained. All DNA samples were then preserved at −80 °C before processing for bacterial community analysis.

The bacterial diversity and community composition of all biofilm samples were measured using the Illumina Miseq sequencing at Suzhou Genewiz Company. Using 30–50 ng DNA as the template, the 16S rRNA genes covering the V3-V4 regions were first amplified from the DNA extracts using the forward primer 347F "CCTACGGRRBGCASCAGKVRVGAAT", and the reverse primer 802R

"GGACTACNVGGGTWTCTAATCC". PCR amplification was conducted in triplicate for each sample using 25 µL PCR reactions mixture containing 2.5 µL TransStart Buffer, 2 µL dNTPs, 2 µL of each primer, 0.2 µL BSA, 0.4 µL FastPfu DNA polymerase, 20 ng DNA template and ddH_2O. PCR was performed using the following conditions: initial denaturation at 95 °C for 3 min, 24 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 90 s, and extension at 72 °C for 10 s. The PCR amplicons were checked by 2% agarose gel electrophoresis and purified using MagPure Gel Pure DNA Mini Kit (Magen, Guangzhou, China). The purified amplicons were pooled and paired-end sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA, USA) at a read length of 2 × 300 bp.

After 16S rRNA sequencing, the reads were sorted to the samples according to barcodes, and the barcodes and primers were then removed. The low-quality reads were discarded, including the reads which did not exactly match the primer, the reads containing ambiguous character (N), a sequence length <200 bp, and reads with an average quality score <20. Then, chimeric sequences were detected and removed by comparing the sequences with the reference database (RDP Gold database) [32] using UCHIME algorithm [33]. The high-quality sequences were clustered into operational taxonomic units (OTUs) using the clustering program VSEARCH9 (1.9.6) against the Silva 128 16S rRNA database with 97% sequence identity threshold. The Ribosomal Database Program (RDP) classifier was used to assign a taxonomic category to all OTUs at a confidence threshold of 0.8. The 16S rRNA gene sequences were submitted to the National Centre for Biotechnological Information (NCBI) Sequence Read Archive database under the accession numbers MH889163-MH890450.

2.5. Statistical Analysis

We evaluated differences in habitat characteristics, physico-chemical features, bacterial diversity and richness in different stream types (forest, urban restored and degraded) using one-way analysis of variance [34], followed by the Tukey's HSD post-hoc test for comparison of means. Pearson correlation coefficients were used to explore relationships between environmental parameters and all microbial variables. Differences were accepted as significant at the $p = 0.05$ level. These statistical analyses were performed in the R statistical environment [35].

Based on the results of the operational taxonomic units (OTUs) analysis, α-diversity indices (Shannon-Weiner index; Chao1 richness) were calculated in QIIME1.9.1 [36]. Non-metric multi-dimensional scaling (NMDS) plot was performed to display β-diversity based on Euclidean dissimilarities between each samples using the 'vegan' package [37] within the R statistical Environment [35]. Analysis of similarities (ANOSIM) was then performed to evaluate the bacterial community similarity among three river types using the vegan package. Venn diagrams were drawn to analyze overlapped and unique OTUs of each sample based on cluster analysis of OTUs. Metastats [38] was performed to detect the differentially abundant taxonomic groups at phylum and genus levels between different river types. The relationships between the bacterial community and environmental parameters (pH, turbidity, DO, TN, TP, TOC, NH_4-N, NO_3-N and COD) were assessed using redundancy analysis (RDA) within Canoco 4.5 for Windows [39].

3. Results

3.1. Habitat Characteristics

There was a significant difference in canopy cover among the different river types ($F_{2,6} = 13.435$, $p = 0.006$); canopy cover was significantly greater in forest rivers, intermediate in degraded rivers, and lowest in restored rivers. Forests and restored rivers had greater diversity of river bed habitat types than degraded rivers. In the forest and restored rivers, riffles, pools, islands were commonly found whereas in the degraded rivers only pools, and a few islands were observed. In terms of substrate composition, the Shannon diversity (H') of the substrate (ranging from 0 to 1.13) was significantly greater in the forest and restored rivers ($p = 0.001$) and lowest in degraded rivers. Only granules were found in degraded rivers, whereas the restored and forest rivers had boulders (forest-only), cobbles,

pebbles and granules. Degraded sites had much smaller substrates, whereas restored and forest rivers had bigger substrates.

3.2. Effects of Habitat Restoration on Physico-Chemical Properties of Stream Water

Physico-chemical values (Table 1) revealed no significant differences among river types for river width ($F_{2,6}$ = 0.336) and mean depth ($F_{2,6}$ = 0.791), and no difference in the surface water for pH ($F_{2,6}$ = 1.815), NH_4-N ($F_{2,6}$ = 1.533), NO_3-N ($F_{2,6}$ = 0.374), TN ($F_{2,6}$ = 2.708), TP ($F_{2,6}$ = 0.042) and COD ($F_{2,6}$ = 5.069). However, significant differences were observed in surface water properties among the stream types for DO ($F_{2,6}$ = 7.398, p = 0.024), turbidity ($F_{2,6}$ = 7.69, p = 0.022), TOC ($F_{2,6}$ = 17.86, p = 0.003) and Chl-*a* ($F_{2,6}$ = 8.94, p = 0.016). The forest and restored rivers had similar concentrations of DO, and both had significantly greater DO concentrations than the degraded rivers ($p < 0.05$) (Figure 2A). The turbidity in degraded rivers was much greater than forest rivers (p = 0.018), while no differences were observed between forest rivers and restored rivers, restored rivers and degraded rivers ($p > 0.1$) (Figure 2B). Degraded rivers and restored rivers had greater TOC concentrations than forest rivers (p = 0.002 and p = 0.027, respectively). Although no significant difference was detected when comparing restored rivers with degraded rivers ($p > 0.1$), a reduction in TOC concentration was observed (Figure 2C). In terms of Chl-*a*, no differences were detected when comparing forest rivers with restored rivers and degraded rivers ($p > 0.1$), whereas rivers under restoration had a much higher Chl-*a* concentration than degraded rivers (p = 0.013) (Figure 2D).

3.3. Effects of Habitat Restoration on Bacterial Community Composition

A total of 3,300,566 reads were obtained from the 27 samples. After filtering, denoising, and chimera removal, 1,650,283 high-quality 16S rRNA gene-reads were obtained, ranging from 48,473 to 69,662 reads per sample. Mean OTUs and α-diversity values (Table 2) showed that bacterial diversity measured by Shannon diversity index (H') was different between the river types ($F_{2,6}$ = 14.067, p = 0.005), being significantly greater in degraded rivers ($F_{2,6}$ = 6.98, p = 0.004) than restored rivers, whereas no distinct difference was found between restored rivers and forest rivers with respect to bacterial diversity (Figure 2F). Bacterial richness (Chao 1 Index) varied from 629 to 874, however, no significant differences were detected among river types for bacterial richness (Figure 2E).

The NMDS analysis produced a stress value <0.094, indicating that the ordination produced a good summary of the observed distances between samples with obvious clustering (Figure 3). The bacterial community structures among all three river types were distinct from each other (R = 0.508, p = 0.001) as shown by analysis of similarities (ANOSIM) (Table 3). Although there was some overlap between restored and degraded rivers, the bacterial community composition was significantly different (R = 0.256, p = 0.008) and there was a clear shift in bacterial community composition along the first axes from degraded to restored rivers, and from restored to forest rivers.

Table 1. Mean values of physico-chemical variables in different types of rivers within the Anji City Region, PRC. The values represent the mean ± standard error of three replicate samples.

River Type	Width (m)	Mean Depth (cm)	Dissolved Oxygen (mg/L)	pH	Turbidity	NH_4-N (mg/L)	NO_3-N (mg/L)	TN (mg/L)	TP (mg/L)	Chemical Oxygen Demand (mg/L)	Total Organic C (mg/L)	Chlorophyll *a* (mg/L)
Forest	8.83 ± 1.64	35.87 ± 7.97	14.16 ± 0.80	7.33 ± 0.11	0.62 ± 0.14	0.02 ± 0.01	1.06 ± 0.13	1.99 ± 0.21	0.18 ± 0.02	2.44 ± 0.15	0.48 ± 0.16	0.61 ± 0.23
Restored	13.17 ± 3.09	28.13 ± 7.22	13.14 ± 0.65	7.64 ± 0.14	3.52 ± 0.85	0.08 ± 0.02	1.13 ± 0.40	2.74 ± 0.77	0.17 ± 0.02	3.35 ± 0.76	2.81 ± 0.32	1.22 ± 0.19
Degraded	11.57 ± 5.72	22.87 ± 3.86	7.91 ± 1.52	7.38 ± 0.11	22.81 ± 14.93	1.37 ± 1.19	0.79 ± 0.40	4.01 ± 0.76	0.18 ± 0.05	8.82 ± 3.40	6.70 ± 2.21	0.20 ± 0.09

Table 2. Mean values of microbial diversity in different types of rivers within the Anji City Region, PRC. The values represent the mean ± standard error of three replicate samples.

River Type	Observed OTUs	Unique OTUs	Diversity Indices	
			Chao 1 Value	Shannon-Weiner Index
Forest	604.11 ± 38.87	14.67 ± 0.88	715.45 ± 36.27	6.42 ± 0.12
Restored	585.00 ± 19.86	5.67 ± 3.18	708.84 ± 21.18	5.89 ± 0.15
Degraded	666.89 ± 69.17	30.00 ± 14.80	769.73 ± 72.81	6.98 ± 0.17

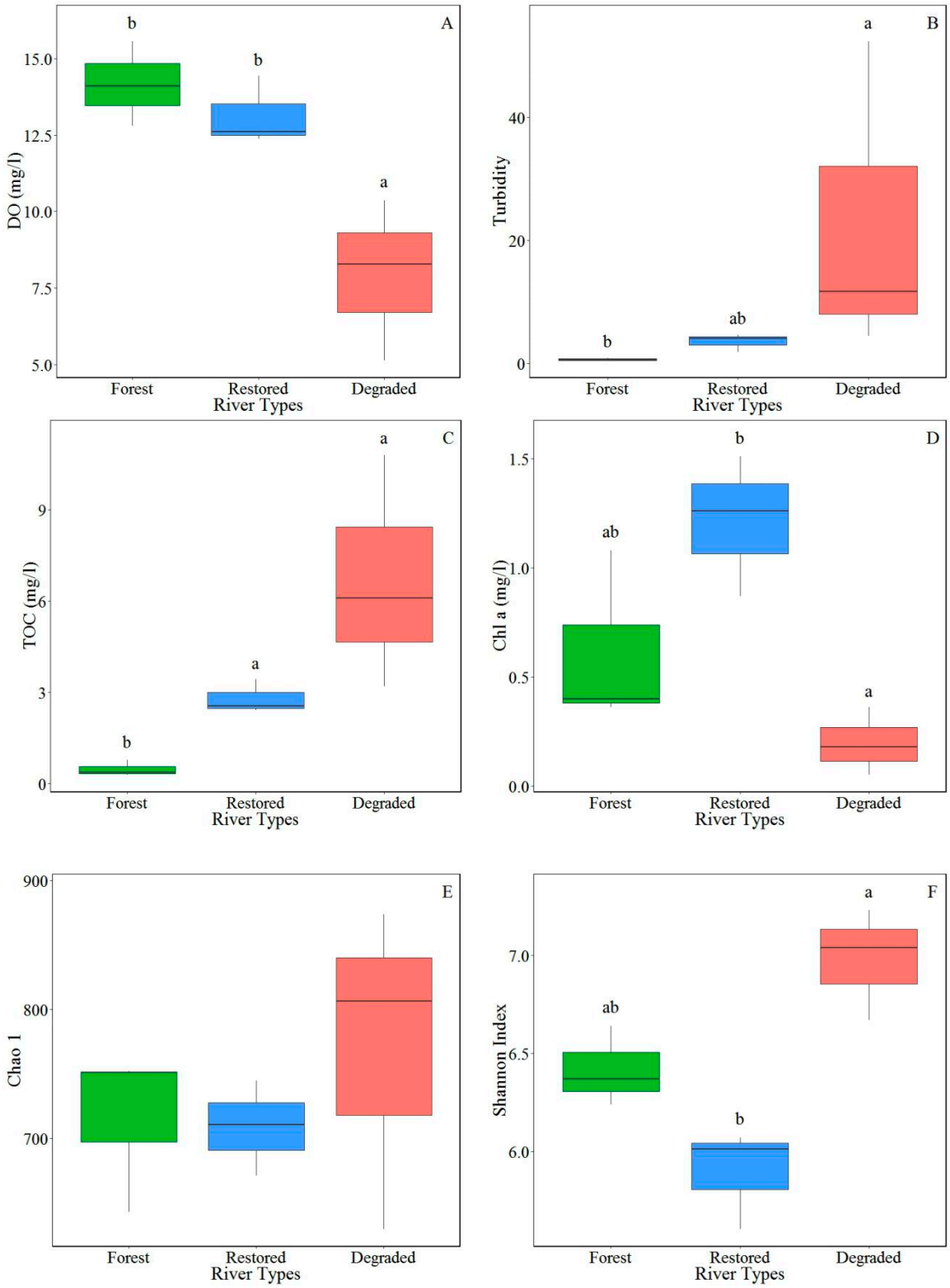

Figure 2. Boxplots representing the variance of physico-chemical parameters (**A**) dissolved oxygen (DO), (**B**) turbidity, (**C**) total organic carbon (TOC), (**D**) Chl-*a* and bacterial α-diversity (**E**) bacterial richness (Chao 1 Index), (**F**) bacterial diversity (Shannon Index) in forested, restored and degraded rivers within the Anji City Region, PRC. Black line: median value; box: quartile interval; whiskers: minimum and maximum value. Different lowercase letters indicate the significant difference observed at the $p = 0.05$ level.

Table 3. Analysis of similarities (ANOSIM) of biofilm bacterial communities in contrasting river types within the Anji City Region, PRC.

River-Type Comparison	ANOSIM	
	R	*p*
Forest vs. Degraded	0.645	0.001
Forest vs. Restored	0.733	0.001
Restored vs. Degraded	0.256	0.008

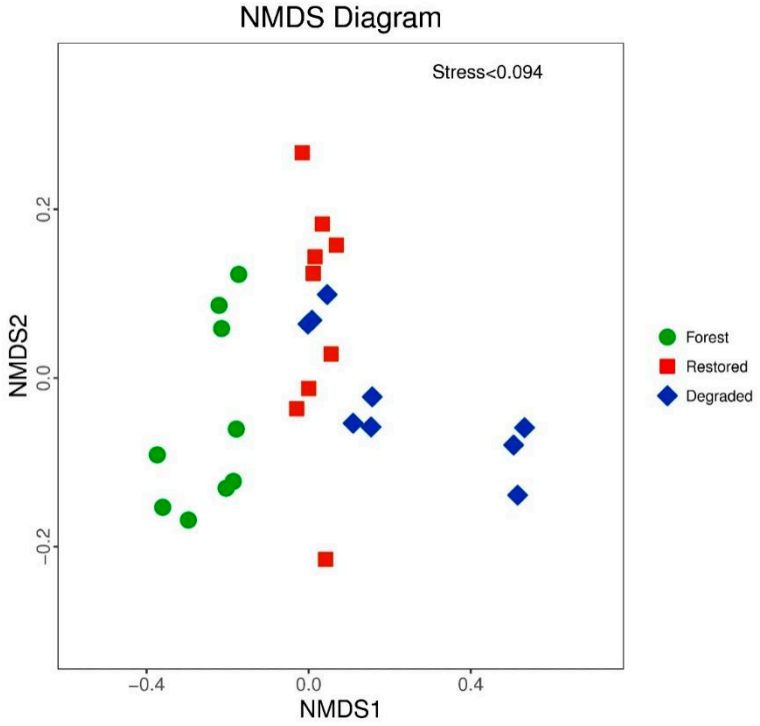

Figure 3. Non-metric multi-dimensional scaling (NMDS, stress < 0.094) ordination of biofilm bacterial communities in forested, restored and degraded rivers within the Anji City Region, PRC within the Anji City Region, PRC.

In total, 383 OTUs were detected, 232 OTUs (61%) of which were universally present from biofilms in all rivers, and the three types of rivers contained 11.5% (forested), 4% (restored) and 23% (degraded) unique OTUs, respectively (Figure 4). The degraded rivers had a greater percentage of unique OTUs, including the members of the orders Rhodocyclales, Cytophagales, Sphingobacteriales, however, no statistical differences were detected among river types for unique OTUs ($F_{2,6} = 2.81$).

The relative abundance of the bacterial community was calculated respectively both at the phylum and genus levels. At the phylum level (Figure 5A), Proteobacteria was the most abundant phylum in all rivers, followed by Bacteroidetes, Firmicutes, Cyanobacteria, Verrucomicrobia, Acidobacteria and Actinobacteria. Rivers in the forest and after restoration had a greater Proteobacteria abundance than degraded rivers ($p = 0.050$, $p = 0.049$, respectively), while no difference was detected between forest and restored rivers ($p > 0.05$). The relative abundance of bacteria in the phylum Bacteroidetes, a taxa commonly assumed to be specialized in degrading high molecular weight (HMW) compounds [40], was slightly greater in degraded rivers than forest rivers ($p = 0.064$), while, no differences of Bacteroidetes were observed when comparing forest rivers with restored rivers, and restored rivers with degraded rivers ($p > 0.01$).

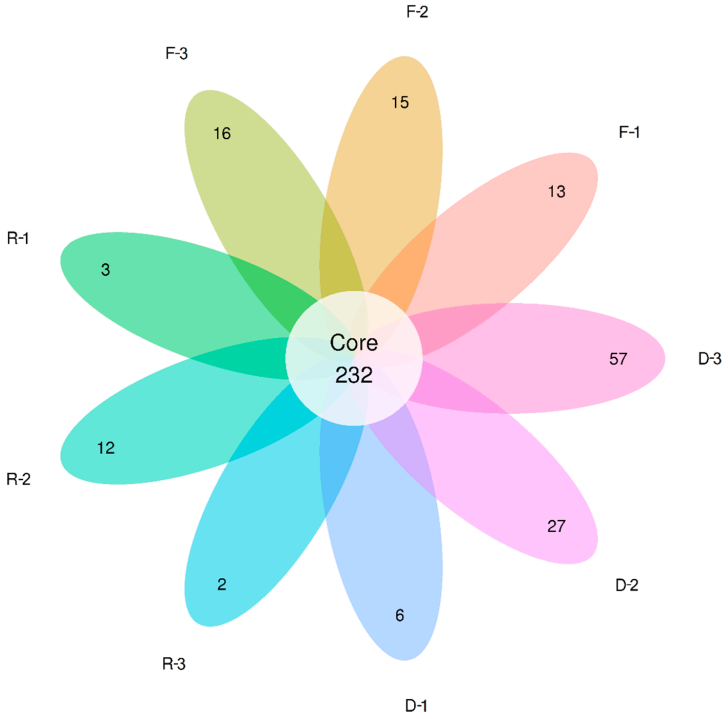

Figure 4. Venn diagram showing the number of unique and shared operational taxonomic units (OTUs) among biofilms in forested (F), restored (R) and degraded (D) rivers within the Anji City Region, PRC.

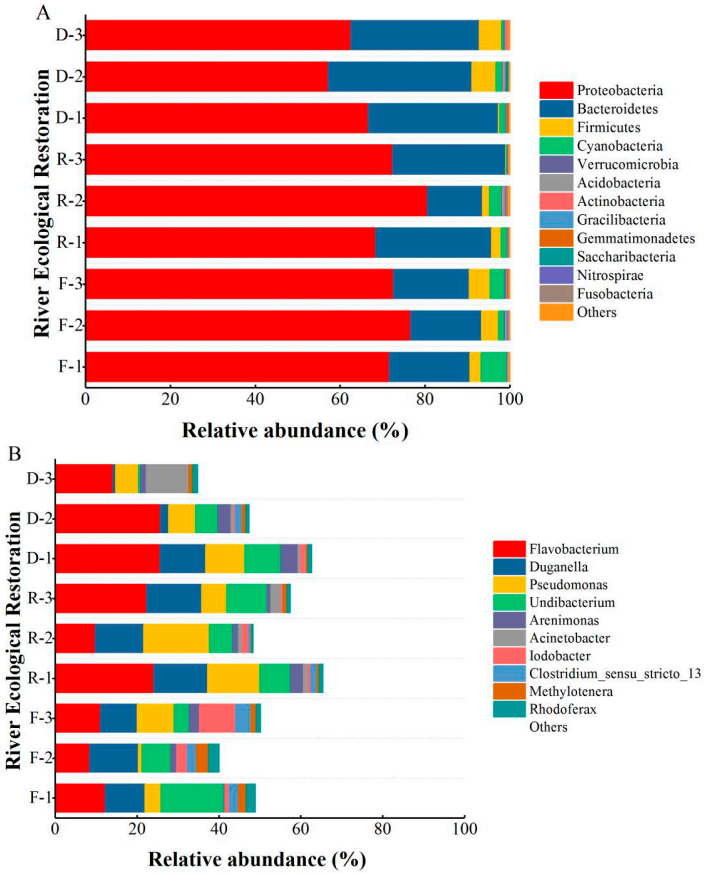

Figure 5. Relative abundance of bacterial community at Phylum (**A**) and Genus level (**B**) in forested (F), restored (R) and degraded (D) rivers within the Anji City Region.

In terms of relative abundance at the genus level, *Flavobacterium*, *Duganella*, *Pseudomonas*, *Undibacterium* and *Arenimonas* were commonly distributed in all studied rivers (Figure 5B). Degraded rivers showed significant numbers of reads allocated to *Flavobacterium* ($p = 0.001$), *Arenimonas* ($p = 0.026$) and *Acinetobacter* ($p = 0.001$). Forest rivers had a higher relative abundance of *Duganella* ($p = 0.022$), *Indobacter* ($p = 0.010$), *Clostridium_sensu_stricto_13* ($p = 0.006$), *Methylotenera* ($p = 0.001$) and *Rhodoferax* ($p = 0.007$) than degraded rivers. Among restored rivers, a greater relative abundance of *Flavobacterium*, *Pseudomonas*, *Acinetobacter* and a lower relative abundance of *Indobacter*, *Clostridium_sensu_stricto_13*, *Methylotenera* and *Rhodoferax* ($p < 0.05$) was found when comparing restored rivers with forest rivers. Restored rivers had a greater relative abundance of *Duganella* ($p = 0.023$) than degraded rivers. No difference in genus abundance was found between restored and degraded rivers for other taxa.

3.4. Correlation between Bacterial Community Composition and Environmental Variables

Bacterial richness (OTUs) showed a positive correlation with water turbidity and a negative correlation with TP concentration ($p = 0.049$, $p = 0.032$, respectively). Bacterial diversity showed a strong positive correlation with water turbidity ($p = 0.006$), COD ($p = 0.023$), and TOC concentration ($p = 0.019$), and was negatively correlated with substrate diversity ($p = 0.033$). The relationship between environmental parameters and the total bacterial community composition was further evaluated by constrained redundancy analysis (RDA), which produced eigenvalues for the first two axes of 0.322 and 0.159, respectively (Figure 6). The environmental variables explained 48.1% of bacterial community structure variance. The biofilm bacterial assemblages in forest rivers were positively correlated with substrate diversity (r = 0.156), and Chl-*a* concentrations (r = 0.828), and were negatively affected by NH_4-N (r = −0.621) and COD (r = −0.629) of surface water. The reverse pattern was found for biofilms in the degraded rivers, COD (r = 0.999), TOC (r = 0.984), NH_4-N (r = 0.738) and TN (r = 0.635) in the surface water presented as major factors linking to the bacterial structure in degraded rivers. For the restored rivers, the bacterial samples showed positive correlations with DO (r = 0.571) and substrate diversity (r = 0.652) and was affected negatively by COD (r = −0.522) and NH_4-N (r = −0.526), though the correlations were not as strong as the forest rivers.

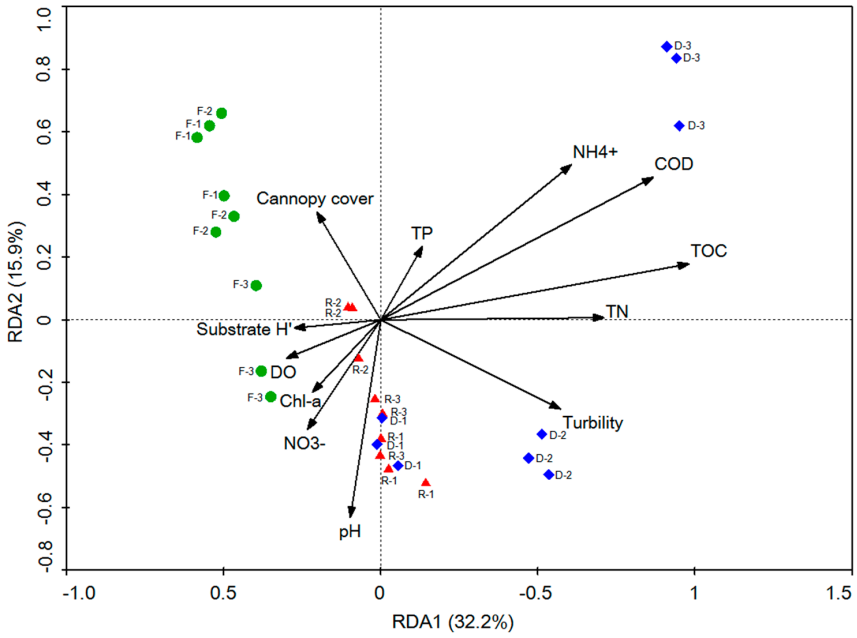

Figure 6. Relationship between the biofilm bacterial community and environmental variables in forested (F, circles), restored (R, triangles) and degraded (D, diamonds) rivers within the Anji City Region, PRC.

4. Discussion

Rehabilitation of aquatic biota, through habitat restoration, is now being implemented around the world to prevent further damage and mitigate existing freshwater degradation [41]. Accumulating evidence has linked aquatic rehabilitation to reducing nitrogen, phosphorus and organic matter concentrations, and thereafter to improved conditions for macro-invertebrate and fish populations [6,15,42]. Microbial communities are often ignored in stream restoration studies yet they are crucial for supporting aquatic ecosystem processes and functions with key roles in driving organic matter and nutrient cycling [43]. It is, therefore, imperative that we obtain a better understanding of the underlying mechanisms of microbe-mediated processes. In this study, therefore, we described the bacterial community composition including those involved in important ecological functions in restored rivers, and compared them with both degraded urban sites and "pristine" reference forest sites; to do this we used high-throughput 16S rRNA gene amplicon sequencing method. The results showed clear differences in the structure of biofilm microbial communities among these three main river ecosystems, and these differences were strongly correlated to the changes in habitat and physico-chemical characteristics in these river groups. This finding is consistent with the results of surveys in New Zealand and the USA, showing that local environmental conditions, rather than spatial factors, such as latitude or elevation, best predicted the variance of community composition and diversity [44,45]. Although the differences in the bacterial community here were mainly led by the variance in habitat and environmental characteristic in the rivers, the longitudinal natural changes in rivers may account for some of the environmental and biological variations observed [46].

4.1. Habitat Restoration Impact on Physico-Chemical Properties of Stream Water

The consistent input of pollutants from both point and diffuse sources in the urban (pre-restored) rivers caused high enrichment of TOC. Habitat restoration led to a reduction in TOC, and a significant increase in DO in the surface water of the restored rivers. These results are consistent with habitat restoration experiments in the Zenne River in Belgium [47]. Essentially, habitat restoration improved conditions by reducing TOC and increasing DO, suggesting that organic pollutants entering the degraded river were removed through habitat restoration. There was no difference in DO concentration between restored and reference forest rivers, suggesting that habitat restoration improved the physico-chemical environment of restored rivers.

4.2. Impact of Habitat Restoration on the Bacterial Community

The diversity and composition of bacterial communities change according to habitat characteristics [48], hence, rehabilitation methods and the intensity of application should affect both the composition and diversity of microbial communities. Here, no differences were detected among river types for bacterial richness, and a significant decline in bacterial diversity was detected in restored rivers compared to degraded rivers. This is consistent with studies in wastewater treatment plant (WWTP) effluent in both urban and rural areas where a reduced diversity of biofilm bacteria has been detected [49,50]. The difference in bacterial diversity might reflect the physico-chemical variables of surface water in the different river types. Dissolved inorganic nitrogen, dissolved organic carbon and hydrological variability has been demonstrated to be the most important environmental factors affecting biofilm responses [51]. In this study, the increase of DO concentration caused by habitat restoration might lead to the development of aerobic microbial community and higher efficiencies of chemical oxygen demand (COD) removal through oxidative decomposition [52]. The decline in organic carbon quality could also influence the abundance of biofilm bacteria [51,53], which might have led to the decrease in heterotrophic anaerobic microorganisms that rely on organic resources, which lead to the decline of bacterial diversity in rivers after habitat restoration. Epilithic bacterial populations can also be affected indirectly by inorganic nutrients via the influence of nutrients on algal biomass [54,55].

Distinct bacterial communities were detected in each of the river types, a dissimilar composition was found between (i) forest rivers and degraded rivers, (ii) forest rivers and restored rivers, and (iii) restored rivers and degraded rivers. These differences were strongly correlated with the changes in habitat substrate diversity, and physico-chemical characteristics (DO, TOC and COD) of these river types. The results from this study suggest that the differences in bacterial community compositions were mainly caused by the variations in habitat and habitat-specific physico-chemical characteristics [48,56]. Rivers with diverse substrates may provide more dynamic surface and a higher degree of resource heterogeneity within the microhabitats for biofilms, shaping distinct bacterial communities in forest and restored rivers from the microbiome in degraded rivers. The variations in physico-chemical attributes (e.g., TOC) in the forest and restored rivers might lead to the difference in bacterial community composition between these two river types. Moreover, the bacteria clustered in the restored rivers were distributed between the bacteria in the degraded and forest rivers, indicating that they were moving in the correct direction, i.e., towards the reference forest rivers. There was, however, some overlap between the restored and degraded rivers, indicating that there was still a legacy effect of the previous degraded state. Overall, the degraded rivers possessed significantly greater bacterial diversity than the restored rivers. Hence, restoration to "pristine" conditions will take longer than seven years, and further studies are needed to determine exactly how long.

Compared with forest rivers, degraded rivers had a slightly greater abundance of Bacteroidetes, a member of phylum specialized in degrading high molecular weight (HMW) compounds, and possessed significantly higher relative abundance of *Flavobacterium*, *Arenimonas* and *Acinetobacter*, which are capable of metabolizing/mineralizing organic compounds [57–59], and a remarkably low abundance of *Duganella*, *Indobacter*, *Methylotenera*, *Rhodoferax* and *Clostridium_sensu_stricto_13*; these genera are major players in cycling of carbon compounds in the environment [60,61], and organic matter utilization [62]. This suggests that the degraded rivers with a high TOC load and limited DO have a distinct impact on the microbial community, shaping the microbiome with a greater ability to degrade/mineralize high molecular weight (HMW) compounds in degraded rivers; this ability differentiates these degraded rivers from the forest ones.

The restored rivers, however, had a greater relative Proteobacteria abundance than degraded rivers; this phylum is often found in nutrient-poor conditions with a low TOC [47]. Moreover, *Duganella* genus, which utilized organic compounds, but required oxygen to survive [63], was greater in restored rivers compared to the degraded ones. This may imply that along with the establishment of more diverse substrates and aerobic and sub-aerobic system in the restored rivers, habitat restoration shifted the dominant components of the bacterial community that mineralize and degrade organic matter to bacteria that utilize organic matter for growth. At the same time, there is also a shift from species that occur in predominantly anaerobic conditions to aerobic conditions. This is consistent with the RDA results, where the bacterial community in the degraded rivers was strongly correlated to organic pollutants TOC and COD, whereas, for restored rivers, the bacterial community only showed weak positive correlations with substrate diversity and DO in the surface water.

In terms of the relationship between restored rivers and forest rivers, no significant differences in bacterial diversity, bacterial richness, and relative abundance of the Proteobacteria and Bacteroidetes were found. However, restored rivers possessed a lower abundance of *Indobacter*, *Methylotenera*, *Rhodoferax* and *Clostridium_sensu_stricto_13* than forest rivers. Moreover, the *Flavobacterium*, *Pseudomonas* and *Acinetobacter* were found in greater abundance in degraded rivers were much greater in restored rivers compared to forest rivers. This suggests that restored rivers still possess species that degrade/mineralize the high concentrations of organic compounds that persist even after restoration. In summary, our results highlight effective dissolved oxygen enhancement, organic pollutants reduction trends, and alongside changes in the microbial community during river habitat restoration. However, restored rivers still have a long way to go to recover the natural status of pristine rivers, and continued monitoring is needed to measure the time scale required for the restored sites to attain the reference standards.

5. Conclusions

We examined the effect of habitat restoration on microbial community composition in biofilms using high-throughput 16S rRNA gene amplicon sequencing. The results showed that habitat restoration altered the bacterial community structure in a positive manner in the degraded rivers. Habitat restoration induced a lower bacterial diversity, but a greater abundance of genera that degrade organic pollutants; these changes might be attributed to the status of dissolved oxygen and total organic carbon variables in the surface water. These results suggest that applying habitat restoration approaches to restore urban rivers by enhancing habitat heterogeneity, which can, in turn, alter the physico-chemical characteristics and stimulate the processing of organic pollutants through the variation of microbial community composition, which was moving in the right direction. Habitat restoration is, therefore, an efficient way for the switching of microbial community composition for sustainable freshwater restoration and management. It will take longer than seven years for degraded rivers to attain a similar ecosystem quality as the reference sites, and continued studies are needed to measure the time scale required for the recovery.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4441/11/6/1244/s1, Table S1: Detailed location data and habitat information for the nine study sites within the Anji City Region, PRC; Habitat information include canopy cover, habitat types, substrate composition and substrate Shannon index (H'). F = forest streams; R = restored streams; D = degraded streams.

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